



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 39/39, 39/00, 39/29, 39/015, A61P 31/12, 31/04, 33/06, 35/00, 37/08		A2	(11) International Publication Number: WO 00/23105
			(43) International Publication Date: 27 April 2000 (27.04.00)
(21) International Application Number: PCT/EP99/07764 (22) International Filing Date: 8 October 1999 (08.10.99) (30) Priority Data: 9822703.6 16 October 1998 (16.10.98) GB 9822709.3 16 October 1998 (16.10.98) GB 9822712.7 16 October 1998 (16.10.98) GB (71) Applicant (for all designated States except US): SMITHKLINE BEECHAM BIOLOGICALS S.A. [BE/BE]; Rue de l'Institut 89, B-1330 Rixensart (BE). (72) Inventor; and (75) Inventor/Applicant (for US only): GARCON, Nathalie [FR/BE]; SmithKline Beecham Biologicals S.A., Rue de l'Institut 89, B-1330 Rixensart (BE). (74) Agent: DALTON, Marcus, Jonathan, William; SmithKline Beecham, Corporate Intellectual Property, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).			(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: ADJUVANT SYSTEMS AND VACCINES			
(57) Abstract The present invention provides vaccine and adjuvant formulation comprising an immunostimulant and a metal salt. The immunostimulant is adsorbed onto a particle of metal salt and the resulting particle is essentially devoid of antigen.			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

ADJUVANT SYSTEMS AND VACCINES

The present invention relates to improved vaccines, adjuvant systems, and processes for the preparation of such vaccines and adjuvant systems. In particular, the vaccines and adjuvant systems of the present invention comprise metallic salts and additional immunostimulants such as monophosphoryl lipid A, or derivatives thereof, Quil A or derivatives thereof, or immunostimulatory oligonucleotides such as CpG.

Aluminium salts are well known in the art as providing a safe excipient with adjuvant activity. The mechanism of action of these adjuvants are thought to include the formation of an antigen depot such that antigen may stay at the site of injection for up to 3 weeks after administration, and also the formation of antigen/metallic salt complexes which are more easily taken up by antigen presenting cells. In addition to aluminium, other metallic salts have been used to adsorb antigens, including salts of zinc, calcium, cerium, chromium, iron, and beryllium. The hydroxide and phosphate salts of aluminium are the most common.

Vaccine formulations containing aluminium salts, antigen, and additional immunostimulant are known in the art. Such formulations induced greater immune responses in comparison with those stimulated by aluminium salts and antigen alone. Formulation of these vaccine preparations have previously involved a specific manufacturing procedure, since it was believed that in order for optimal immune responses to occur, the antigen must be adsorbed onto the same aluminium salt particle as the immunostimulant. In this way when antigen is taken up by an antigen presenting cell, the co-adsorbed immunostimulant exerts its stimulatory activity directly onto that same antigen presenting cell.

Aluminium based vaccine formulations wherein the antigen and the immunostimulant 3-de-O-acylated monophosphoryl lipid A (3D-MPL), are adsorbed onto the same particle are described in EP 0 576 478 B1, EP 0 689 454 B1, and EP 0 633 784 B1. In these cases then antigen is first adsorbed onto the aluminium salt followed by the adsorption of the immunostimulant 3D-MPL onto the same aluminium salt particles. Such processes first involve the suspension of 3D-MPL by sonication in a water bath until the particles reach a size of between 80 and 500 nm.

The antigen is typically adsorbed onto aluminium salt for one hour at room temperature under agitation. The 3D-MPL suspension is then added to the adsorbed antigen and the formulation is incubated at room temperature for 1 hour, and then kept at 4°C until use.

5 The formulation processes of the prior art provide for potent vaccines from an immunological point of view, however, they do contain several commercial disadvantages. In order for a vaccine to be suitable for human administration, the process must be uniform and be subject to Good Manufacturing Practice (GMP) control, and Quality Control (QC). In some cases the processes of the prior art
10 provide a vaccine wherein all of the antigen, or antigens, are adsorbed onto the same particle of metallic salt. The process is then complicated by the requirement for the 3D-MPL to be adsorbed onto the same metallic particle. This may be particularly problematical in the case of combination vaccines containing multiple antigens (whose adsorption may be dependent on the affinity of each antigen to the
15 particular metallic salt at a given pH). The processes of the prior art may have problems, depending on which antigens are present, in reproducibility and vaccine QC. Furthermore, if anything undesired occurs with the QC of one particular antigen, or an occurrence which may result in the contamination of the vaccine, this may result in the waste all of the individual components, and not just the particular
20 antigen in which the problem occurred. Moreover, in some circumstances combination vaccines may require the sequential addition of the antigens, such a process being hugely time consuming and expensive. The processes of the prior art may, therefore, be complex, difficult to control, and expensive.

 Surprisingly, the present inventors have discovered that it is not necessary to
25 adsorb antigen and the immunostimulant onto the same particle. In contrast to the accepted thinking in the art, it has been found that good vaccines may be produced when antigen is adsorbed onto particular metallic salt particles which are discrete from those metallic salt particles which are associated with the immunostimulant.

 The improved process comprises the adsorption of immunostimulant, onto a
30 metallic salt particle, followed by the adsorption of the antigen onto another metallic salt particle, followed by the mixing of the discrete metallic particles to form a vaccine. The present invention also provides for an adjuvant composition an

immunostimulant, adsorbed onto a metallic salt particle, characterised in that the metallic salt particle is substantially free of other antigen. Furthermore, vaccines are provided by the present invention and are characterised in that the immunostimulant is adsorbed onto particles of metallic salt which are substantially free from other
5 antigen, and in that the particles of metallic salt which are adsorbed to the antigen are substantially free of other immunostimulant.

Accordingly, the present invention provides an adjuvant formulation comprising immunostimulant which has been adsorbed onto a particle of a metallic salt, characterised in the composition is substantially free of other antigen.
10 Moreover, this adjuvant formulation is an intermediate which is required during the process of the present invention, for the manufacture of a vaccine. Accordingly there is provided a process for the manufacture of a vaccine comprising admixing the adjuvant composition of the invention with an antigen. Preferably, the antigen has been pre-adsorbed onto a metallic salt. Said metallic salt may be identical or
15 similar to the metallic salt which is adsorbed onto the immunostimulant.

The present invention further provides for a vaccine composition comprising immunostimulant adsorbed onto a first particle of a metallic salt, and antigen adsorbed onto a metallic salt, characterised in that first and second particles of metallic salt are different.

20 Alternatively, vaccines which form part of the present invention comprise two major populations of complexes, a first complex comprising (a) immunostimulant adsorbed onto a metallic salt particle, characterised in that said metallic salt particle is substantially free of antigen; and a second complex comprising (b) antigen adsorbed onto a metallic salt particle. Also the vaccine composition can comprising
25 two major populations of complexes, a first complex comprising (a) immunostimulant adsorbed onto a metallic salt particle, characterising in that said metallic salt particle is substantially free of antigen; and a second complex comprising (b) antigen adsorbed onto a metallic salt particle, characterised in that said metallic salt particle is substantially free of immunostimulant.

30 The metallic salts present in these two major populations of complexes may be identical or different. Furthermore, in the case of a combination vaccine, wherein a

plurality of different antigens may be present, the second complex (described above) may comprise a plurality of antigens adsorbed onto different metallic particles.

The definition of substantially free of other antigens, in relation to this invention, is where not more than 20% by mass of the total material capable of adsorbing to the particle of metallic salt is an other antigen, preferably not more than 10%, and most preferably not more than 5%. Alternatively, the substantially free at immunostimulant, in relation to this invention, is where not more than 20% by mass of the total material capable of adsorbing to the particle of metallic salt is immunostimulant, preferably not more than 10%, and most preferably not more than 5%. Routine assays, apparent to the man skilled in the art, could be used to determine whether the antigen and immunostimulant, are adsorbed onto different discrete particles, including but not limited to separation of the vaccine into distinct fractions by free flow of the formulation within an electric field, or techniques such as sedimentation rate analysis which are particularly suited to non-particulate antigens, followed by assaying for the immunostimulant or antigen in the fractions.

Also provided in the present invention is a kit comprising one container having immunostimulant adsorbed onto a metallic salt; and a second container having antigen, preferably said antigen being adsorbed onto a metallic salt.

The process of the present invention is especially useful when commercial scale quantities of combination vaccines are required. Combination vaccines are single dose vaccines which contain more than one antigen from more than one pathogen. Such vaccines may reduce the number of vaccinations required to induce protection against many pathogens and diseases.

For example, if a vaccine comprises $\text{Al}(\text{OH})_3$, 3D-MPL, and the antigens V, W, X, Y, Z, previous processes involve formulating the antigens and the 3D-MPL onto the same particle of $\text{Al}(\text{OH})_3$. Such prior art processes require that V, W, X, Y, Z are adsorbed onto the $\text{Al}(\text{OH})_3$, followed by the addition of free 3D-MPL onto each of the pre-adsorbed antigen complexes.

In contrast, the formulation process of the present invention antigens V, W, X, Y, Z are each individually adsorbed onto separate particles of $\text{Al}(\text{OH})_3$ in separate containers. 3D-MPL is also adsorbed onto $\text{Al}(\text{OH})_3$ in another container. The vaccine is then formed by the simple admixing of material taken from each of the separate

containers. In this case the particles of AlOH_3 which is associated with the 3D-MPL may be discrete from the particles of AlOH_3 which are associated with the antigens.

Alternatively, the present invention provides a process of making a vaccine
5 comprising an immunostimulant, antigen and a metallic salt, comprising:

1. Adsorbing antigen to a first particle of metallic salt,
2. Adsorbing the immunostimulant to a second particle of a metallic salt, and
3. admixing the products of steps 1 and 2 above.

The present invention provides for a process for the manufacture of vaccines
10 which overcome the problems present in the prior art. Each individual antigen-metallic salt complex may be subject to GMP controls, and should any untoward contamination of a particular antigen-metallic salt preparation then the integrity of other antigens and immunostimulant adjuvant will not be compromised. Surprisingly, and in contrast to the accepted thinking in the art, vaccines produced
15 by the process of the present invention are as potent as those prepared using the process of the prior art.

The definition of immunostimulant within the meaning of this invention can be described as a natural or synthetic compound which has known adjuvant activity, which adjuvant activity derives from the direct or indirect stimulatory effect of the
20 compound upon the cells of the immune system itself and not through other non-stimulatory effects such as a depot effect or targeting to the immune system. Examples of such immunostimulants are described in a chapter in "Vaccine Design - the subunit and adjuvant approach" (Edited by Powell, M.F. and Newman, M.J.; 1995, Pharmaceutical Biotechnology (Plenum Press, New York and London, ISBN
25 0-306-44867-X) entitled "Compendium of vaccine adjuvants and excipients" by Powell, M.F. and Newman M.. These immunostimulants include which are within the present invention include: bacterially derived compounds, like Monophosphoryl lipid A or derivatives thereof; plant derived saponins or derivatives thereof, for example Quil A; or immunostimulatory oligonucleotides such as CpG, block
30 copolymers, cholera toxin, immunostimulatory cytokines such as GM-CSF and IL-1, polyriboA and polyriboU, and Muramyl tripeptide (MTP).

Monophosphoryl lipid A is a bacterially derived compound with adjuvant activity, and is a preferred immunostimulant for use in the present invention. This toxic compound has been altered to form less toxic derivatives, one such derivative is 3 De-O-acylated monophosphoryl lipid A (termed 3D-MPL or d3-MPL, to indicate that position 3 of the reducing end glucosamine is de-O-acylated). For preparation of 3D-MPL, see GB 2 220 211 A. Chemically it is a mixture of 3-deacylated monophosphoryl lipid A with 3, 4, 5 or 6 acylated chains. Preferably in the compositions of the present invention small particle MPL is used. Small particle MPL has a particle size such that it may be sterile-filtered through a 0.22µm filter. Such preparations are described in International Patent Application No. WO 94/21292. Further improvements are described in GB 9807933.8 which discloses stable preparations of 3D-MPL consisting of the tri and tetra acyl congeners.

GB 2 220 211A mentions that the endotoxicity of the previously used enterobacterial lipopolysaccharides (LPS) is reduced while the immunogenic properties are conserved. However GB 2 220 211 cited these findings merely in connection with bacterial (Gram negative) systems.

Another preferred immunostimulant for use in the present invention is Quil A and its derivatives. Quil A is a saponin preparation isolated from the South American tree *Quilaja Saponaria Molina* and was first described by Dalsgaard *et al.* in 1974 ("Saponin adjuvants", Archiv. für die gesamte Virusforschung, Vol. 44, Springer Verlag, Berlin, p243-254) to have adjuvant activity. Purified fragments of Quil A have been isolated by HPLC which retain adjuvant activity without the toxicity associated with Quil A (EP 0 362 278), for example QS7 and QS21 (also known as QA7 and QA21). Particular formulations of QS21 have been described which are particularly preferred, these formulations further comprise a sterol (WO96/33739).

CpG is an immunostimulatory oligonucleotide with known adjuvant properties (WO 96/02555). The CpG sequences preferred within the context of this invention are: (TCC ATG AGC TTC CTG ACG TT, Krieg 1826), (TCT CCC AGC GTG CGC CAT, Krieg 1758), and TCG TCG TTT TGT CGT TTT GTC GTT.

The present invention relates to the particular formulation process and characteristics of the adjuvant, and thus can be utilised with a wide variety of antigens. The vaccines of the present invention can be used for priming and boosting doses, and used for the induction of immune responses to, and protection from infection mediated by, a wide variety of antigens. Also the present invention provides for a method of eliciting an immune response to an antigen comprising the use of a vaccine comprising a metallic salt, immunostimulant, and antigen, wherein the immunostimulant is adsorbed onto particles of metallic salt which are discrete from those metallic salt particles which are adsorbed to the antigen. Some of the pathogens and antigens are listed below.

Viral hepatitis, caused by the A, B, C, D, and E hepatitis viruses, is a very common viral illness. Via the B and C viruses, in particular, it is also responsible for many cases of liver cancer. Thus the development of effective vaccines is critical and, despite notable successes, is still an on-going task. A review on modern hepatitis vaccines, including a number of key references, may be found in the Lancet, May 12th 1990 at page 1142 ff (Prof A.L.W.F. Eddleston). See also 'Viral Hepatitis and Liver Disease' (Vyas, B.N., Dienstag, J.L., and Hoofnagle, J.H., eds, Grune and Stratton. Inc. (1984) and 'Viral Hepatitis and Liver Disease' (Proceedings of the 1990 International Symposium, eds F.B. Hollinger, S.M. Lemon and H. Margolis, published by Williams and Wilkins).

As used herein the expression 'hepatitis B antigen' is used to refer to any antigenic material derived from a hepatitis B virus which may be used to induce immunity to the virus in humans.

Infection with hepatitis B virus (HBV) is a widespread problem but vaccines which can be used for mass immunisation are now available, for example the product 'Engerix-B' (SmithKline Beecham plc) which is obtained by genetic engineering techniques.

The preparation of Hepatitis B surface antigen (HBsAg) is well documented. See, for example, Harford et al in Develop. Biol. Standard 54, page 125 (1983), Gregg et al in Biotechnology. 5, page 479 (1987), EP-A- 0 226 846, EP-A-0 299 108 and references therein.

As used herein the expression 'Hepatitis B surface antigen' or 'HBsAg' includes any HBsAg antigen or fragment thereof displaying the antigenicity of HBV surface antigen. It will be understood that in addition to the 226 amino acid sequence of the HBsAg S antigen (see Tiollais et al, Nature, 317, 489 (1985) and references therein) HBsAg as herein described may, if desired, contain all or part of a pre-S sequence as described in the above references and in EP-A- 0 278 940. In particular the HBsAg may comprise a polypeptide comprising an amino acid sequence comprising residues 12-52 followed by residues 133-145 followed by residues 175-400 of the L-protein of HBsAg relative to the open reading frame on a Hepatitis B virus of ad serotype (this polypeptide is referred to as L*; see EP 0 414 374). HBsAg within the scope of the invention may also include the preS1-preS2 -S polypeptide described in EP 0 198 474 (Endotronics) or analogues thereof such as those described in EP 0 304 578 (Mc Cormick and Jones). HBsAg as herein described can also refer to mutants, for example the 'escape mutant' described in WO 91/14703 or European Patent Application Publication Number 0 511 855 A1, especially HBsAg wherein the amino acid substitution at position 145 is to arginine from glycine.

Normally the HBsAg will be in particle form. The particles may comprise for example S protein alone or may be composite particles, for example (L*,S) where L* is as defined above and S denotes the S-protein of HBsAg. The said particle is advantageously in the form in which it is expressed in yeast.

The component affording protection against Hepatitis A is preferably the product known as 'Havrix' (SmithKline Beecham Biologicals) which is a killed attenuated vaccine derived from the HM-175 strain of HAV [see 'Inactivated Candidate Vaccines for Hepatitis A' by F.E. Andre, A. Hepburn and E.D'Hondt (1980), Prog. Med. Virol. Vol 37, pages 72-95 and the product monograph 'Havrix' published by SmithKline Beecham Biologicals (1991).

Thus, in a preferred embodiment of the present invention a combination vaccine comprising HBsAg and Hepatitis A antigen is provided. Also, provided by the present invention is a process for the production of a hepatitis A and B combination vaccine, and a product derived from that process.

Other combination vaccines are available on the market including the Infanrix™ range, made by SmithKline Beecham Biologicals. Such vaccines are based on a "core" combination of Diphtheria toxin, Tetanus toxin, and *B. pertussis* antigens. This vaccine comprises a pertussis component (either killed whole cell *B. pertussis* or acellular *pertussis* which typically consists of two antigens - PT and FHA, and often 69kDa, optionally with one or both agglutinin 2 or agglutinin 3). Such vaccines are often referred to as DTPw (whole cell) or DTPa (acellular).

Particular combination vaccines within the scope of the invention include:

Diphtheria-Tetanus- Pertussis-Hepatitis B (DTP-HB)

10 Diphtheria-Tetanus-Hepatitis B (DT-HB)

Hib-Hepatitis B

DTP-Hib-Hepatitis B

IPV (inactivated polio vaccine)- DTP-Hib-Hepatitis B

The pertussis component is suitably a whole cell pertussis vaccine or an acellular pertussis vaccine containing partially or highly purified antigens. The above combinations may optionally include a component which is protective against Hepatitis A. Preferably the Hepatitis A component is formalin HM-175 inactivated. Advantageously, the HM-175 is purified by treating the cultured HM-175 with trypsin, separating the intact virus from small protease digested protein by permeation chromatography and inactivating with formalin. Advantageously the Hepatitis B combination vaccine is a paediatric vaccine.

Other combination vaccines of the present invention are disclosed in GB 9805105.5 (SmithKline Beecham Biologicals s.a.), such combination vaccines being specially beneficial for vaccines for adolescents. Preferred combinations are based around a "core" combination of a Hepatitis B antigen (Hep B) and a Herpes Simplex (HSV) antigen. Optionally, to this "core" may be added one or more antigens derived from the following group: Epstein Barr Virus (EBV) antigen, Hepatitis A antigen (Hep A), Human Papilloma Virus (HPV) antigen. These combination vaccines may additionally comprise Varicella Zoster Virus (VZV), Human Cytomegalovirus (HCMV) or toxoplasma antigens.

Preferably the vaccine formulations of the present invention contain an antigen or antigenic composition capable of eliciting an immune response against a

human pathogen, which antigen or antigenic composition is derived from HIV-1, (such as tat, nef, gp120 or gp160), human herpes viruses, such as gD or derivatives thereof or Immediate Early protein such as ICP27 from HSV1 or HSV2, cytomegalovirus ((esp Human)(such as gB or derivatives thereof), Rotavirus

5 (including live-attenuated viruses), Epstein Barr virus (such as gp350 or derivatives thereof), Varicella Zoster Virus (such as gpI, II and IE63), or from a hepatitis virus such as hepatitis B virus (for example Hepatitis B Surface antigen or a derivative thereof), hepatitis A virus, hepatitis C virus and hepatitis E virus, or from other viral pathogens, such as paramyxoviruses: Respiratory Syncytial virus (such as F

10 and G proteins or derivatives thereof), parainfluenza virus, measles virus, mumps virus, human papilloma viruses (for example HPV6, 11, 16, and 18), flaviviruses (e.g. Yellow Fever Virus, Dengue Virus, Tick-borne encephalitis virus, Japanese Encephalitis Virus) or Influenza virus, or derived from bacterial pathogens such as *Neisseria spp.*, including *N. gonorrhoea* and *N. meningitidis* (for example capsular

15 polysaccharides and conjugates thereof, transferrin-binding proteins, lactoferrin binding proteins, PilC, adhesins); *Streptococcus spp.*, including *S. pneumoniae* (for example capsular polysaccharides and conjugates thereof, PsaA, PspA, streptolysin, choline-binding proteins), *S. pyogenes* (for example M proteins or fragments thereof, C5A protease, lipoteichoic acids), *S. agalactiae*, *S. mutans*; *Haemophilus*

20 *spp.*, including *H. influenzae type B* (for example PRP and conjugates thereof), *non typeable H. influenzae* (for example OMP26, high molecular weight adhesins, P5, P6, lipoprotein D), *H. ducreyi*; *Moraxella spp.*, including *M. catarrhalis*, also known as *Branhamella catarrhalis* (for example high and low molecular weight adhesins and invasins); *Bordetella spp.*, including *B. pertussis* (for example pertactin,

25 pertussis toxin or derivatives thereof, filamentous hemagglutinin, adenylate cyclase, fimbriae), *B. parapertussis* and *B. bronchiseptica*; *Mycobacterium spp.*, including *M. tuberculosis* (for example ESAT6, Antigen 85A, -B or -C), *M. bovis*, *M. leprae*, *M. avium*, *M. paratuberculosis*, *M. smegmatis*; *Legionella spp.*, including *L. pneumophila*; *Escherichia spp.*, including enterotoxigenic *E. coli* (for

30 example colonization factors, heat-labile toxin or derivatives thereof, heat-stable toxin or derivatives thereof), enterohemorrhagic *E. coli*, enteropathogenic *E. coli* (for example shiga toxin-like toxin or derivatives thereof); *Vibrio spp.*, including *V.*

- cholera* (for example cholera toxin or derivatives thereof); *Shigella spp.*, including *S. sonnei*, *S. dysenteriae*, *S. flexnerii*; *Yersinia spp.*, including *Y. enterocolitica* (for example a Yop protein), *Y. pestis*, *Y. pseudotuberculosis*; *Campylobacter spp.*, including *C. jejuni* (for example toxins, adhesins and invasins) and *C. coli*;
- 5 *Salmonella spp.*, including *S. typhi*, *S. paratyphi*, *S. choleraesuis*, *S. enteritidis*; *Listeria spp.*, including *L. monocytogenes*; *Helicobacter spp.*, including *H. pylori* (for example urease, catalase, vacuolating toxin); *Pseudomonas spp.*, including *P. aeruginosa*; *Staphylococcus spp.*, including *S. aureus*, *S. epidermidis*; *Enterococcus spp.*, including *E. faecalis*, *E. faecium*; *Clostridium spp.*, including *C. tetani* (for
- 10 example tetanus toxin and derivative thereof), *C. botulinum* (for example botulinum toxin and derivative thereof), *C. difficile* (for example clostridium toxins A or B and derivatives thereof); *Bacillus spp.*, including *B. anthracis* (for example botulinum toxin and derivatives thereof); *Corynebacterium spp.*, including *C. diphtheriae* (for example diphtheria toxin and derivatives thereof); *Borrelia spp.*, including *B.*
- 15 *burgdorferi* (for example OspA, OspC, DbpA, DbpB), *B. garinii* (for example OspA, OspC, DbpA, DbpB), *B. afzelii* (for example OspA, OspC, DbpA, DbpB), *B. andersonii* (for example OspA, OspC, DbpA, DbpB), *B. hermsii*; *Ehrlichia spp.*, including *E. equi* and the agent of the Human Granulocytic Ehrlichiosis; *Rickettsia spp.*, including *R. rickettsii*; *Chlamydia spp.*, including *C. trachomatis* (for example
- 20 MOMP, heparin-binding proteins), *C. pneumoniae* (for example MOMP, heparin-binding proteins), *C. psittaci*; *Leptospira spp.*, including *L. interrogans*; *Treponema spp.*, including *T. pallidum* (for example the rare outer membrane proteins), *T. denticola*, *T. hyodysenteriae*; or derived from parasites such as *Plasmodium spp.*, including *P. falciparum*; *Toxoplasma spp.*, including *T. gondii* (for example SAG2, SAG3, Tg34); *Entamoeba spp.*, including *E. histolytica*; *Babesia spp.*, including *B.*
- 25 *microti*; *Trypanosoma spp.*, including *T. cruzi*; *Giardia spp.*, including *G. lamblia*; *Leshmania spp.*, including *L. major*; *Pneumocystis spp.*, including *P. carinii*; *Trichomonas spp.*, including *T. vaginalis*; *Schistosoma spp.*, including *S. mansoni*, or derived from yeast such as *Candida spp.*, including *C. albicans*; *Cryptococcus*
- 30 *spp.*, including *C. neoformans*.

In one preferred aspect the vaccine formulation of the invention comprises the HIV-1 antigen, gp120, especially when expressed in CHO cells. In a further

embodiment, the vaccine formulation of the invention comprises gD2t as hereinabove defined.

In a preferred embodiment of the present invention vaccines containing the claimed adjuvant comprise the HPV viruses considered to be responsible for genital warts, (HPV 6 or HPV 11 and others), and the HPV viruses responsible for cervical cancer (HPV16, HPV18 and others). Particularly preferred forms of vaccine comprise L1 particles or capsomers, and fusion proteins comprising one or more antigens selected from the HPV 6 and HPV 11 proteins E6, E7, L1, and L2. The most preferred forms of fusion protein are: L2E7 as disclosed in GB 95 15478.7, and proteinD(1/3)-E7 disclosed in GB 9717953.5 (WO99/10375).

Vaccines of the present invention further comprise antigens derived from parasites that cause Malaria. For example, preferred antigens from *Plasmodia falciparum* include RTS,S and TRAP. RTS is a hybrid protein comprising substantially all the C-terminal portion of the circumsporozoite (CS) protein of *P.falciparum* linked via four amino acids of the preS2 portion of Hepatitis B surface antigen to the surface (S) antigen of hepatitis B virus. Its full structure is disclosed in the International Patent Application No. PCT/EP92/02591, published under Number WO 93/10152 claiming priority from UK patent application No.9124390.7. When expressed in yeast RTS is produced as a lipoprotein particle, and when it is co-expressed with the S antigen from HBV it produces a mixed particle known as RTS,S. TRAP antigens are described in the International Patent Application No. PCT/GB89/00895, published under WO 90/01496. A preferred embodiment of the present invention is a Malaria vaccine wherein the antigenic preparation comprises a combination of the RTS,S and TRAP antigens. Other plasmodia antigens that are likely candidates to be components of a multistage Malaria vaccine are *P. falciparum* MSP1, AMA1, MSP3, EBA, GLURP, RAP1, RAP2, Sequestrin, PfEMP1, Pf332, LSA1, LSA3, STARP, SALSA, PfEXP1, Pfs25, Pfs28, PFS27/25, Pfs16, Pfs48/45, Pfs230 and their analogues in *Plasmodium* spp.

The formulations may also contain an anti-tumour antigen and be useful for the immunotherapeutic treatment cancers. For example, the adjuvant formulation finds utility with tumour rejection antigens such as those for prostate, breast, colorectal, lung, pancreatic, renal or melanoma cancers. Exemplary antigens

include MAGE 1 and MAGE 3 or other MAGE antigens for the treatment of melanoma, PRAME, BAGE or GAGE (Robbins and Kawakami, 1996, Current Opinions in Immunology 8, pps 628-636; Van den Eynde et al., International Journal of Clinical & Laboratory Research (submitted 1997); Correale et al.

- 5 (1997), Journal of the National Cancer Institute 89, p293. Indeed these antigens are expressed in a wide range of tumour types such as melanoma, lung carcinoma, sarcoma and bladder carcinoma. Other Tumor-Specific antigens are suitable for use with adjuvant of the present invention and include, but are not restricted to Prostate specific antigen (PSA) or Her-2/neu, KSA (GA733), MUC-1 and carcinoembryonic
10 antigen (CEA). Other antigens have been put forward as being pan-cancer therapeutic antigens including Tyrosinase and Survivin. Accordingly in one aspect of the present invention there is provided a vaccine comprising an adjuvant composition according to the invention and a tumour rejection antigen.

- It is foreseen that compositions of the present invention will be used to
15 formulate vaccines containing antigens derived from *Borrelia sp.*. For example, antigens may include nucleic acid, pathogen derived antigen or antigenic preparations, recombinantly produced protein or peptides, and chimeric fusion proteins. In particular the antigen is OspA. The OspA may be a full mature protein in a lipidated form virtue of the host cell (E.Coli) termed (Lipo-OspA) or a non-
20 lipidated derivative. Such non-lipidated derivatives include the non-lipidated NS1-OspA fusion protein which has the first 81 N-terminal amino acids of the non-structural protein (NS1) of the influenza virus, and the complete OspA protein, and another, MDP-OspA is a non-lipidated form of OspA carrying 3 additional N-terminal amino acids.

- 25 Vaccines of the present invention may be used for the prophylaxis or therapy of allergy. Such vaccines would comprise allergen specific (for example Der p1, and pollen related antigens) and allergen non-specific antigens (for example the stanworth decapeptide).

- The amount of antigen in each vaccine dose is selected as an amount which
30 induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each

dose will comprise 1-1000 μg of antigen, preferably 1-500 μg , preferably 1-100 μg , most preferably 1 to 50 μg . An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or
5 several booster immunisation adequately spaced. Typically for human administration the immunostimulant will be present in the range 1 μg - 1000 μg , preferably 10 μg - 500 μg , more preferably 20-200 μg per dose, more preferably 20-100 μg per dose, and most preferably 10-50 μg per dose.

The present invention further provides for the adjuvants and vaccines of the
10 present invention for use in medicine, specifically as a method of treating a mammal suffering from or susceptible to a pathogenic infection, or cancer, or allergy. Also provided for is the use of the adjuvants and vaccines of the present invention in the manufacture of a immunoprophylactic and immunotherapeutic treatment of viral, bacterial, parasitic infections, allergy, or cancer. The formulations of the present
15 invention maybe used for both prophylactic and therapeutic purposes.

Vaccine preparation is generally described in "Vaccine Design - the subunit and adjuvant approach" Edited by Powell, M.F. and Newman, M.J.; 1995, Pharmaceutical Biotechnology (Plenum Press, New York and London, ISBN 0-306-44867-X)

20 The present invention is illustrated by, but not restricted to, the following examples.

Example 1, *Materials and Methods*

25 *Serology*

Quantification of anti-HBs antibody was performed by ELISA using HBs (Hep 286) as coating antigen. Antigen and antibody solutions were used at 50 μl per well. Antigen was diluted at a final concentration of 1 $\mu\text{g}/\text{ml}$ in PBS and was adsorbed overnight at 4°C to the wells of 96 wells microtiter plates (Maxisorb Immuno-plate,
30 Nunc, Denmark). The plates were then incubated for 1hr at 37°C with PBS containing 1% bovine serum albumin and 0.1% TWEEN 20 (saturation buffer;

100 μ l/well). Two-fold dilutions of sera (starting at 1/100 dilution) in the saturation buffer were added to the HBs-coated plates and incubated for 1 hr 30 min at 37°C. The plates were washed four times with PBS 0.1 % TWEEN 20 and biotin-conjugated anti-mouse IgG1, IgG2a, IgG2b or Ig (Amersham, UK) diluted 1/1000
5 in saturation buffer was added to each well and incubated for 1 hr 30 min at 37°C. After a washing step, streptavidin-biotinylated peroxidase complex (Amersham, UK) diluted 1/5000 in saturation buffer was added for an additional 30 min at 37°C. Plates were washed as above and incubated for 20 min with a solution of o-phenylenediamine (Sigma) 0.04 % H₂O₂ 0.03 % in 0.1 % TWEEN 20, 0.05M citrate
10 buffer pH 4.5. The reaction was stopped with H₂SO₄ 2N and read at 490/630 nm. ELISA titers were calculated from a reference by SoftmaxPro (using a four parameters equation) and expressed in EU/ml.

T cell proliferation

15 2 weeks after the second immunisation, mice were killed, spleens were removed aseptically in pools. Cell suspensions were prepared in RPMI 1640 medium (GIBCO) containing 2 mM L-glutamine, antibiotics, 5x10⁻⁵ M 2-mercaptoethanol, and 1% syngeneic normal mouse serum. Splenic cells were cultured at a final concentration of 2x10⁶ cells/ml in 200 μ l in round-bottomed 96 wells-plates with different
20 concentrations (10-0.03 μ g/ml) of HBs antigen . Each test was carried out in quadruplicate. After 96 hr of culture at 37°C under 5% CO₂, the cells were pulsed for 18hr with ³H-Thymidine (Amersham, UK, 5Ci/mmol) at 0.5 μ Ci/well and then harvested on Unifilter plates (Packard) with a cell harvester. Incorporated radioactivity was measured in a scintillation counter (Topcount, Packard). Results are
25 expressed in cpm (mean cpm in quadruplicate wells) or as stimulation indices (mean cpm in cultures of cells with antigen / mean cpm in cultures of cells without antigen).

Cytokine production

2 weeks after the second immunisation, mice were killed, spleens were removed
30 aseptically in pools (3 pools per group). Cell suspensions were prepared in RPMI 1640 medium (GIBCO) containing 2 mM L-glutamine, antibiotics, 5x10⁻⁵ M 2-mercaptoethanol, and 5% foetal calf serum. Cells were cultured at a final

concentration of 5×10^6 cells/ml in 1ml, in flat-bottomed 24 wells-plates with different concentrations (10-0.1 $\mu\text{g/ml}$) of HBs antigen. Supernatants were harvested 96 hrs later and frozen until tested for the presence of IFN γ and IL-5 by ELISA.

5

IFN γ production

Quantification of IFN γ was performed by ELISA using reagents from Genzyme. Samples and antibody solutions were used at 50 μl per well. 96-wells microtiter plates (Maxisorb Immuno-plate, Nunc, Denmark) were coated overnight at 4°C with 50 μl of hamster anti-mouse IFN γ diluted at 1.5 $\mu\text{g/ml}$ in carbonate buffer pH 9.5. Plates were then incubated for 1hr at 37°C with 100 μl of PBS containing 1 % bovine serum albumin and 0.1 % TWEEN 20 (saturation buffer). Two-fold dilutions of supernatant from in vitro stimulation (starting at 1/2) in saturation buffer were added to the anti- IFN γ -coated plates and incubated for 1 hr 30 at 37°C. The plates were washed 4 times with PBS TWEEN 0.1 % (wash buffer) and biotin-conjugated goat anti-mouse IFN γ diluted in saturation buffer at a final concentration of 0.5 $\mu\text{g/ml}$ was added to each well and incubated for 1 hr at 37°C. After a washing step, AMDEX conjugate (Amersham) diluted 1/10000 in saturation buffer was added for 30 min at 37°C. Plates were washed as above and incubated with 50 μl of TMB (Biorad) for 10 min. The reaction was stopped with H $_2$ SO $_4$ 0.4N and read at 450/630 nm. Concentrations were calculated using a standard curve (mouse IFN γ standard) by SoftmaxPro (four parameters equation) and expressed in pg/ml.

IL-5 production

Quantification of IL-5 was performed by ELISA using reagents from Pharmingen. Samples and antibody solutions were used at 50 μl per well. 96-wells microtiter plates (Maxisorb Immuno-plate, Nunc, Denmark) were coated overnight at 4°C with 50 μl of rat anti-mouse IL-5 diluted at 1 $\mu\text{g/ml}$ in carbonate buffer pH 9.5. Plates were then incubated for 1hr at 37°C with 100 μl PBS containing 1 % bovine serum albumin and 0.1 % TWEEN 20 (saturation buffer). Two-fold dilutions of supernatant from in vitro stimulation (starting at 1/2) in saturation buffer were added to the anti-

IFN γ -coated plates and incubated for 1 hr 30 at 37°C. The plates were washed 4 times with PBS TWEEN 0.1% (wash buffer) and biotin-conjugated rat anti-mouse IL-5 diluted in saturation buffer at a final concentration of 1 μ g/ml was added to each well and incubated for 1 hr at 37°C. After a washing step, AMDEX conjugate (Amersham) diluted 1/10000 in saturation buffer was added for 30 min at 37°C. Plates were washed as above and incubated with 50 μ l of TMB (Biorad) for 15 min. The reaction was stopped with H₂SO₄ 0.4N and read at 450/630 nm. Concentrations were calculated using a standard curve (recombinant mouse IL-5) by SoftmaxPro (four parameters equation) and expressed in pg/ml.

Example 2, Immunogenicity studies in mice

In order to test the concept of MPL on a solid particulate carrier free of antigen, an immunogenicity study was performed in Balb/C mice using various sequences of formulations of HABMPL vaccines:

Table 1, Vaccine formulations

<i>Group</i>	<i>Formulation</i>
1	(HB-AIPO ₄) + 3D-MPL + (HA-Al(OH) ₃)
2	(3D-MPL-Al(OH) ₃) + (HA-Al(OH) ₃) + (HB-AIPO ₄)
3	(3D-MPL-AIPO ₄) + (HA-Al(OH) ₃) + (HB-AIPO ₄)

Description of formulation process:

Group 1, The formulation process of the prior art. The antigen is first adsorbed onto the metallic salt followed by the addition of free 3D-MPL, resulting in the adsorption of the 3D-MPL onto the same particle of metallic salt as the antigen.

Group 2 and 3, The formulation process of the present invention. The 3D-MPL is adsorbed onto one particle of metallic salt, the antigens are adsorbed onto separate particles of metallic salt, followed by the admixture of the pre-adsorbed complexes.

Immunisation scheme

Groups of 10 mice were immunised subcutaneously twice at 4 weeks interval with HAB based formulations (1/10 Human Dose, i.e. HAV 72 ELU, HBs 2 µg, MPL 5 µg). On day 14 post II, lymphoproliferative response and cytokine production

- 5 (IL5/IFN γ) were analysed after *in vitro* restimulation of splenic cells with HBs and HAV. Blood was taken from the retroorbital sinus on day 35 and antibody response to HBs and HAV as well as the isotypic profile induced (HBs only) were monitored by ELISA.

10 *Results*

Humoral responses (Ig and isotypes) were measured by ELISA using HBs as coating antigen for HBV and using the Behring kit for HAV. Only 14 days post II bleeding was analysed.

- 15 **Figure 1** shows anti-HBs Ig antibody responses measured on individual sera and represented as GMT.

Figure 2 shows the isotypic repartition (IgG1, IgG2a and IgG2b) calculated from analysis on pooled sera .

- 20 No differences in antibody titers are observed between group 1 and the novel formulations (groups 2 and 3). Furthermore, the novel formulations (groups 2 and 3) stimulate similar proportions of IgG1 and IgG2a/b isotypes as those stimulated by the formulations of the prior art (group 1).

25 *Cell-mediated immune responses*

Cell-mediated immune responses (lymphoproliferation and IFN γ / IL-5 production) were measured at 14 days post II after *in vitro* restimulation of splenic cells with HBs or HA antigens. For each group of mice, 5 animals were sacrificed and spleens pooled for *in vitro* testing.

30

Figure 3 shows the lymphoproliferation monitored in splenic cells restimulated with HBs.

Figure 4 shows the cytokine production monitored in splenic cells restimulated with HBs.

No differences lymphoproliferative responses can be observed between the
5 formulations.

Strong IFN- γ (+/- 1000 pg/ml) responses were observed with all groups, moreover, no difference in IL-5 production (below 60 pg/ml) is observed between the groups.

10 *Conclusions*

No significant differences in humoral and cell-mediated immune responses to HBsAg were observed between the HABMPL formulation sequences.

15 *Example 3, HSV vaccination of Guinea Pigs.*

The previous example demonstrated the efficacy of the new formulations and processes, with respect to Hepatitis antigens. This example investigated the immunogenicity and protective efficacy of Herpes Simplex Virus gD vaccines formulated with alum and 3D-MPL in the classical process in comparison with the
20 process of the present invention. The two vaccines were compared in the HSV Guinea pig intravaginal protection model.

<i>Group</i>	<i>Formulations</i>	
4	gD2t (20 μ g) + 3D-MPL (50 μ g) + AlOH (500 μ g)	
5	gD2t (20 μ g) + AlOH (400 μ g)	3D-MPL (50 μ g) + AlOH (100 μ g)
6	Untreated	

Experimental protocol

25 Groups of 12 female Hartley guinea pigs were immunised twice at days 0 and 28. At day 57, the animals were challenged intra-vaginally with 105 pfu of HSV2 MS strain (100 μ l). After challenge, animals were monitored daily for clinical signs of primary disease from day 4 to 12. Blood was taken from the retro-orbital sinus at days 14 and 28 after the second immunisation and the anti-gD antibody response
30 (IgG) was monitored by ELISA.

Formulation process

gD2t from HSV2 was produced according to techniques described in WO 92/16231.

5 3D-MPL was purchased from Ribi ImmunoChem Inc., Montana, USA. Al(OH)₃ was purchased from Superfos. Formulations were prepared 15 days before the first injection. All incubations were carried out at room temperature with agitation.

Group 4 Al(OH)₃-based formulations (250 µl/dose): classical way

10

gD2t (5 µg) was adsorbed on 125 µg of Al(OH)₃ for 15 min before MPL addition (12.5 µg). Thirty minutes later, the formulation was buffered with a 10-fold concentrated PBS pH 7.4 solution. After 15 min, 500 µg/ml of phenoxyethanol was added as preservative.

15

H₂O + Al(OH)₃ + Ag-15m-MPL-30m-10xPBS pH 7.4-15m-2 phenoxy

Group 5 Al(OH)₃-based formulations (250 µl/dose): new way

20

gD2t (5 µg) was adsorbed on 100 µg of Al(OH)₃ for 15 min and stocked as a concentrated monobulk. In an other hand, MPL (12.5 µg) was adsorbed on 25 µg of Al(OH)₃ for 30 min and stocked as an other concentrated monobulk.. For the final formulation, the adsorbed gD2t was diluted in H₂O and 10-fold concentrated PBS pH 7.4. Fifteen minutes later, adsorbed MPL was added before phenoxyethanol addition as preservative.

25

Al(OH)₃ + Ag

Al(OH)₃ + MPL

H₂O + 10xPBS pH 7.4 + Ads gD2t-15m-Ads MPL-15m-2 phenoxy

30

Sample Quantification

Quantification of anti-gD antibody was performed by Elisa using gD 43B318 as coating antigen. Antigen and antibody solutions were used at 50 µl per well.

Antigen was diluted at a final concentration of 1 µg/ml in PBS and was adsorbed

overnight at 4°C to the wells of 96 wells microtiter plates (Maxisorb Immuno-plate, Nunc, Denmark). The plates were then incubated for 1 hr at 37°C with PBS containing 1% bovine serum albumin and 0.1% Tween 20 (saturation buffer). Two-fold dilutions of sera in the saturation buffer were added to the gD-coated plates and
5 incubated for 1 hr 30 min at 37°C. The plates were washed four times with PBS 0.1% Tween 20 and biotin-conjugated anti-guinea pig IgG (Amersham, UK) diluted 1/10000 in saturation buffer was added to each well and incubated for 1 hr 30 min at 37°C. After a washing step, streptavidin-biotinylated peroxidase complex (Amersham, UK) diluted 1/1000 in saturation buffer was added for an additional 30
10 min at 37°C. Plates were washed as above and incubated for 20 min with a solution of o-phenylenediamine (Sigma) 0.04% H₂O₂ 0.03% in 0.1% tween 20 0.05M citrate buffer pH4.5. The reaction was stopped with H₂SO₄ 2N and read at 490/630 nm. ELISA titers were calculated from a reference by SoftmaxPro (using a four parameters equation) and expressed in EU/ml.

15

Statistical Analysis

Statistical analyses were performed on serology data using UNISTAT:

The protocol applied for one way analysis of variance can be briefly described as follows:

- 20 1) Log transformation of data.
- 2) Kolmogorov Smirnov test on each population (group) in order to verify the normality.
- 3) Hartley and Cochran tests in order to verify the homogeneity of variance between the different populations (groups).
- 25 4) Analysis of variance on selected data: 14 days post II or 28 days post II data.

Results

30 *Serology*

Figure 5, shows anti-gD IgG antibody responses measured at post II on individual sera.

No striking difference in antibody titers is observed between either formulations at day 14 post II (17090-18508 EU/ml for GMT) or 28 days post II (10227-11965 EU/ml for GMT). One way analysis of variance was performed separately on anti-gD IgG titres raised by either vaccine formulation, from both time-points after Log transformation of data. No statistically significant differences between either formulations were detected (p- values = 0.7397 and 0.5078 for respectively 14 days post II and 28 days post II data).

10 *Protection from Disease*

Protection against primary disease was evaluated between 4 to 12 days post challenge, by comparing several parameters in vaccinated and untreated animals:

- The percentage of animals with and without lesions (vaginal or external).
- 15 • The primary infection index (PI) calculated for each group as follows:

$$\Sigma (\text{score maximum} \times \text{incidence expressed in } \%)$$
- The sum of lesion scores (day 4 to 12) expressed as median and the number of animals showing lesions (N).
- The mean cumulative scores calculated for each group between day 4 and 12

20 *Table 2 summarises the lesion parameters*

Group	Animals without lesions (%)	Vaginal lesions (%)	External lesions (%)	Primary infection index*	Lesion severity (n)**
4	66.7	25	8.3	29.2 -97%	1 (4)
5	83.3	16.7	0	8.3 -99%	0.5 (2)
6	11.1	0	88.9	844.4	28.3 (8)

* Sum of lesion scores for the days 4 to 12 post-infection (animals without lesions were not considered). Lesion scores: no lesion (0), vaginal lesions (0.5 or 1), external skin vesicles (2, 4, 8 or 16).

** Primary infection index = (max score I) × (incidence %); with I = 0, 0.5, 1, 2, 4, 8 or 16.

Figure 6 shows the cumulative lesion score curves after HSV challenge.

5 A high percentage of vaccinated animals did not develop any lesion (66% to 83%) or developed vaginal lesions. In comparison, 89 % of the animals of the control group showed external lesions.

10 A strong reduction of primary infection index was observed in vaccinated animals (97% to 99%). This was accompanied with a very low lesion severity was recorded for the vaccinated groups (median = 0.5 or 1) compared to the untreated group (median = 28).

15 As shown by the curves of cumulative scores both groups (4 and 5) gave a very good and comparable level of protection against primary disease

Conclusion

Old and new processes for vaccine HSV vaccine formulation were compared. No statistically significant difference was observed between the two processes in either IgG titres or in protection against primary disease.

20

Example 4, HPV vaccination of mice

Various sequences of formulations (AIOH or AIPO4 based) of Human Papiloma Virus E7 antigen and 3D-MPL were compared regarding their capacity to induce antigen-specific humoral responses. Comparable Ig titers are obtained with
25 formulations with mixed adsorption of 3D-MPL and proteinD1/3-E7 on the same carrier (way 1) and formulations where 3D-MPL is adsorbed separately on a carrier free of antigen (way 2). Protein D 1/3 E7 were prepared according to the procedure of WO 99/10375. The antigen and MPL formulations were either AIOH-based or AIPO4-based.

30 Antigen and 3D-MPL were adsorbed sequentially to the same particles of Aluminium salt (way 1), or separate adsorptions were made prior to mixing (way 2).

Groups of 10 mice were immunized using the following formulations (description in Material and Methods):

Group	Description	Formulation
7	ProtD1/3 E7-AIOH	5 μ g ProtD 1/3 E7 adsorbed on AIOH
8	ProtD1/3 E7-AIOH/MPL	5 μ g ProtD 1/3 E7 adsorbed on AIOH with MPL (way 1)
9	ProtD1/3 E7-AIOH/ (ALPO4/MPL)	5 μ g ProtD 1/3 E7 adsorbed on AIOH combined with MPL adsorbed on AlPO4 (way 2)
10	ProtD1/3 E7-AlPO4	5 μ g ProtD 1/3 E7 adsorbed on AlPO4
11	ProtD1/3 E7-AlPO4/MPL	5 μ g ProtD 1/3 E7 adsorbed on AlPO4 with MPL (way 1)
12	ProtD1/3 E7-AlPO4/(AIOH/MPL)	5 μ g ProtD 1/3 E7 adsorbed on AlPO4 combined with MPL adsorbed on AIOH (way 2)
13	ProtD1/3 E7 o/w	5 μ g ProtD 1/3E7 formulated in oil in water emulsion 3D-MPL/QS21

Mice were immunized twice at a 21-day interval by the intramuscular route. Sera were collected on Day 35 (14 days post II) and analysed for the presence of E7 specific antibodies (see Material and Methods). Formulations were prepared 5 days before the first injection. All incubations were carried out at room temperature with agitation.

10 **I. Al-based formulations (50 μ l/dose): classical way (way 1)**

PD1/3E7 (5 μ g) was adsorbed on 50 μ g of Al(OH)₃ or AlPO₄ for 30 min before MPL addition (5 μ g). Thirty minutes later, the formulation was buffered with a 10-fold concentrated PO₄,NaCl pH 6.8 solution. After 15 min. 50 μ g/ml of thiomersal was added as preservative.

H₂O + Al + Ag-30m-MPL-30m-10xPNpH6.8-15m-Thio

II. Al-based formulations (50 μ l/dose): new way (way 2)

PD1/3E7 (5 μ g) was adsorbed on 10 μ g of Al(OH)₃ or AlPO₄ for 30 min and stocked as concentrated monobulks. In an other hand, MPL (5 μ g) was adsorbed on 20 μ g of Al(OH)₃ or AlPO₄ for 30 min and stocked as others concentrated monobulks. For the final formulations, the adsorbed antigen was diluted in H₂O and 10-fold concentrated PO₄, NaCl pH 6.8 solution before addition of the adsorbed MPL and of the rest of Al (20 μ g). Thirteen minutes later, 50 μ g/ml of thiomersal was added as preservative.

10 Al + Ag

Al + MPL

H₂O + 10xPN pH 6.8 + Ads PD1/3E7 + Ads MPL + Al-30m-Thio

SEROLOGY

- 15 Quantification of anti-E7 antibody was performed by Elisa using E7 (Bollen) as coating antigen. Antigen and antibody solutions were used at 50 μ l per well. Antigen was diluted at a final concentration of 3 μ g/ml in carbonate buffer pH9.5 and was adsorbed overnight at 4°C to the wells of 96 wells microtiter plates (Maxisorb Immuno-plate, Nunc, Denmark). The plates were then incubated for 1hr at 37°C with PBS containing 1% bovine serum albumin and 0.1% Tween 20 (saturation buffer). Two-fold dilutions of sera (starting at 1/100 dilution) in the saturation buffer were added to the E7-coated plates and incubated for 1 hr 30 min at 37°C. The plates were washed 3 times with PBS 0.1% Tween 20 and biotin-conjugated anti-mouse (IgG1, IgG2a or IgG2b or) IgGtot (Amersham, UK) diluted 1/5000 in saturation buffer was added to each well and incubated for 1 hr 30 min at 37°C. After a washing step, streptavidin-biotinylated peroxydase complex (Amersham, UK) diluted 1/5000 in saturation buffer was added for an additional 30 min at 37°C. Plates were washed as above and incubated for 10 min with TMB (tetra-methyl-benzidine). The reaction was stopped with H₂SO₄ 4N and read at 450 nm. Midpoint dilutions were calculated by SoftmaxPro (using a four parameters equation).

Results

The anti-E7 Ig titers measured on pooled sera by ELISA expressed in EU/ml are as follows

	E7-AIOH based formulations	E7-AIPO4 based formulations
Alum	4434	1651
Alum / MPL	10780	12666
Alum / (Alum/MPL)	13390	15495

- 5 Comparable titers are obtained when comparing the AIOH based formulations or the AIPO4 based formulations. When MPL is added to the AIOH or AIPO4 formulations, the titers reached are above 10,000 EU/ml as compared to less than 5,000 EU/ml for the Al formulations. Comparable titers are obtained with both formulation sequences.

10

Various sequences of formulations (AIOH or AIPO4 based) of antigen and MPL were compared regarding their capacity to induce the production of antigen-specific antibodies:

- 15 All formulations containing MPL induce higher levels of E7-specific Ig than Alum formulations. Comparable Ig titers are obtained with formulations with mixed adsorption of MPL and pD1/3-E7 on the same carrier (way 1) and formulations where MPL is adsorbed separately on a carrier free of antigen (way 2).

Claims

1. An adjuvant composition comprising an immunostimulant adsorbed onto a metallic salt particle, characterised in that the metallic salt particle is substantially free of other antigen.
2. An adjuvant composition as claimed in claim 1, wherein the metallic salt particle is a salt of aluminium, zinc, calcium, cerium, chromium, iron, or berilium.
3. An adjuvant composition as claimed in claims 1 or 2, wherein the metallic salt is a phosphate or hydroxide.
4. An adjuvant composition as claimed in any one of claims 1 to 3, wherein the metallic salt is aluminium hydroxide or aluminium phosphate.
5. An adjuvant composition as claimed in any one of claims 1 to 4, wherein the immunostimulant is monophosphoryl lipid A.
6. An adjuvant composition as claimed in claim 5, wherein the derivative of monophosphoryl lipid A is 3-de-O-acylated monophosphoryl lipid A.
7. An adjuvant composition as claimed in any one of claims 1 to 4, wherein the immunostimulant is QS21.
8. An adjuvant composition as claimed in any one of claims 1 to 4, wherein the immunostimulant is CpG containing oligonucleotide.
9. A process for the manufacture of a vaccine composition comprising admixing the adjuvant composition described in claim 1, with antigen.
10. A process for the manufacture of a vaccine composition as claimed in claim 9, characterised in that the antigen is adsorbed onto a metallic salt particle.
11. A process as claimed in any one of claims 9 or 10, wherein the antigen is selected from the group comprising: antigens derived from Human Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2, Human cytomegalovirus, Dengue virus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium or Toxoplasma, IgE peptides, Der p1, pollen related antigens; or Tumor associated antigens (TMA), MAGE, BAGE, GAGE, MUC-1, Her-2 neu, LnRH(GnRH), CEA, PSA, KSA, or PRAME.

12. A vaccine composition comprising two major populations of complexes, a first complex comprising (a) an immunostimulant adsorbed onto a metallic salt particle, characterised in that said metallic salt particle is substantially free of antigen; and a second complex comprising (b) antigen adsorbed onto a metallic salt particle.
13. A vaccine composition comprising two major populations of complexes, a first complex comprising (a) an immunostimulant adsorbed onto a metallic salt particle, characterised in that said metallic salt particle is substantially free of antigen; and a second complex comprising (b) antigen adsorbed onto a metallic salt particle, characterised in that said metallic salt particle is substantially free of monophosphoryl lipid A, or derivative thereof.
14. A vaccine composition as claimed in claims 12 or 13, wherein the metallic salt present in the first and second complexes are identical.
15. A vaccine composition as claimed in any one of claims 12 to 14, wherein the second complex comprises a plurality of sub-complexes, each sub-complex comprising a different antigen adsorbed onto a metallic particle.
16. A vaccine composition as claimed in any one of claims 12 to 15, wherein the metallic salt is a salt of aluminium, zinc, calcium, cerium, chromium, iron, or berilium.
17. A vaccine composition as claimed in claim 16 wherein the metallic salt is a phosphate or hydroxide.
18. A vaccine composition as claimed in claim 17 wherein the metallic salt is aluminium hydroxide or aluminium phosphate.
19. A vaccine composition as claimed in any one of claims 12 to 18, wherein the immunostimulant is 3-de-O-acylated monophosphoryl lipid A.
20. A vaccine composition as claimed in any one of claims 12 to 18, wherein the immunostimulant is QS21.
21. A vaccine composition as claimed in any one of claims 12 to 18, wherein the immunostimulant is CpG.
22. A vaccine composition as claimed in any one of claims 12 to 21, wherein the antigen is selected from the group comprising: Human Immunodeficiency Virus, Varicella Zoster virus, Herpes Simplex Virus type 1, Herpes Simplex virus type 2,

Human cytomegalovirus, Dengue virus, Hepatitis A, B, C or E, Respiratory Syncytial virus, human papilloma virus, Influenza virus, Hib, Meningitis virus, Salmonella, Neisseria, Borrelia, Chlamydia, Bordetella, Plasmodium or Toxoplasma, stanworth decapeptide, Der p1, pollen related antigens; or cancer associated antigens, MAGE, BAGE, GAGE, MUC-1, Her-2 neu, LnRH(GnRH);

5 CEA, PSA, tyrosinase, Survivin, KSA, or PRAME.

23. A vaccine composition as claimed in claim 22, wherein the antigen is a combination of Hepatitis A antigen and Hepatitis B antigen.

24. A vaccine composition as claimed in claim 22, wherein the plasmodium

10 antigen is one or more antigens selected from the following group: RTS,S and TRAP.

25. A vaccine composition as claimed in claim 22 for use in medicine.

26. Use of vaccine composition as claimed in claim 22, for the manufacture of an immunotherapeutic treatment of viral, bacterial, parasitic infections, allergy, or

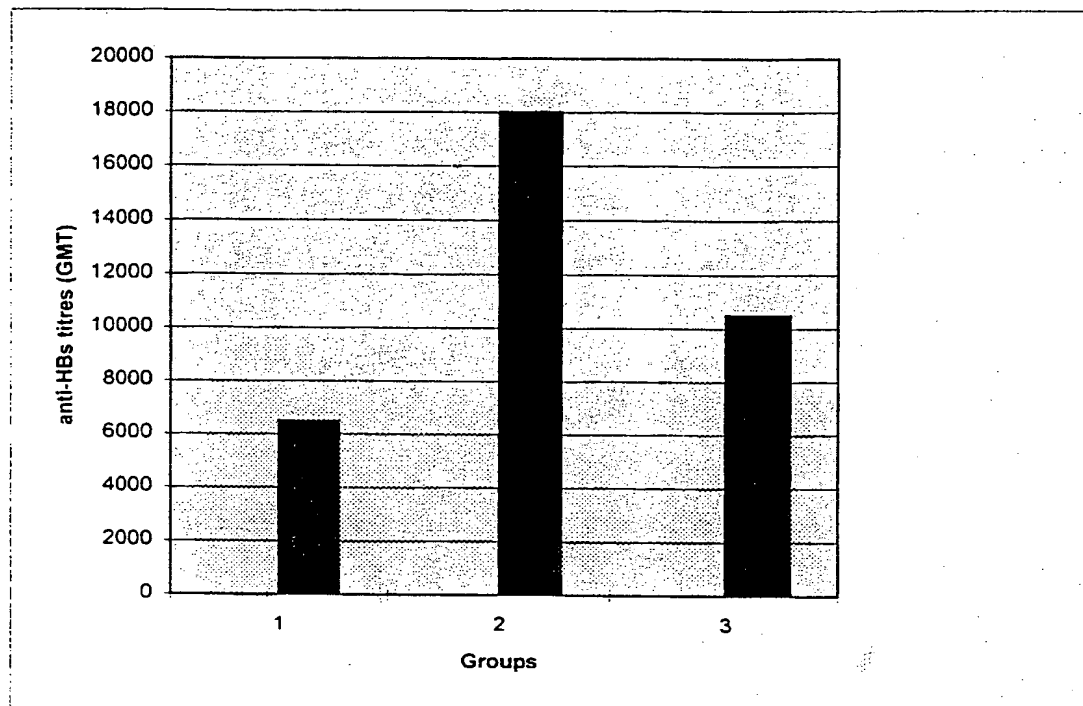
15 cancer.

27. A method of treating a mammal suffering from or susceptible to a pathogenic infection, or cancer, or allergy, comprising the administration of a safe and effective amount of a vaccine composition according to claim 22.

26. A kit comprising two containers, one container having monophosphoryl lipid

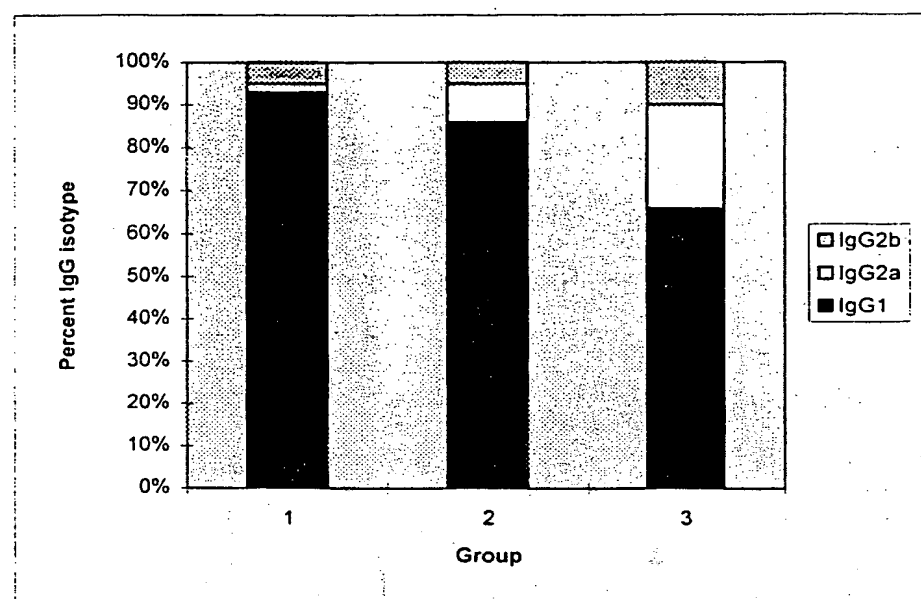
20 A, or derivative thereof, adsorbed onto a metallic salt; and the second container having antigen adsorbed onto a metallic salt.

Figure 1 shows anti-HBs Ig antibody responses measured on individual sera and represented as GMT.



Group	ELISA Titres (GMT)
1	6495
2	18006
3	10496

Figure 2 shows the isotypic repartition (IgG1, IgG2a and IgG2b) calculated from analysis on pooled sera .



Group	IgG1 %	IgG2a %	IgG2b %
1	93	2	5
2	86	9	5
3	66	24	10

Figure 3, shows the lymphoproliferation monitored in splenic cells restimulated with HBs.

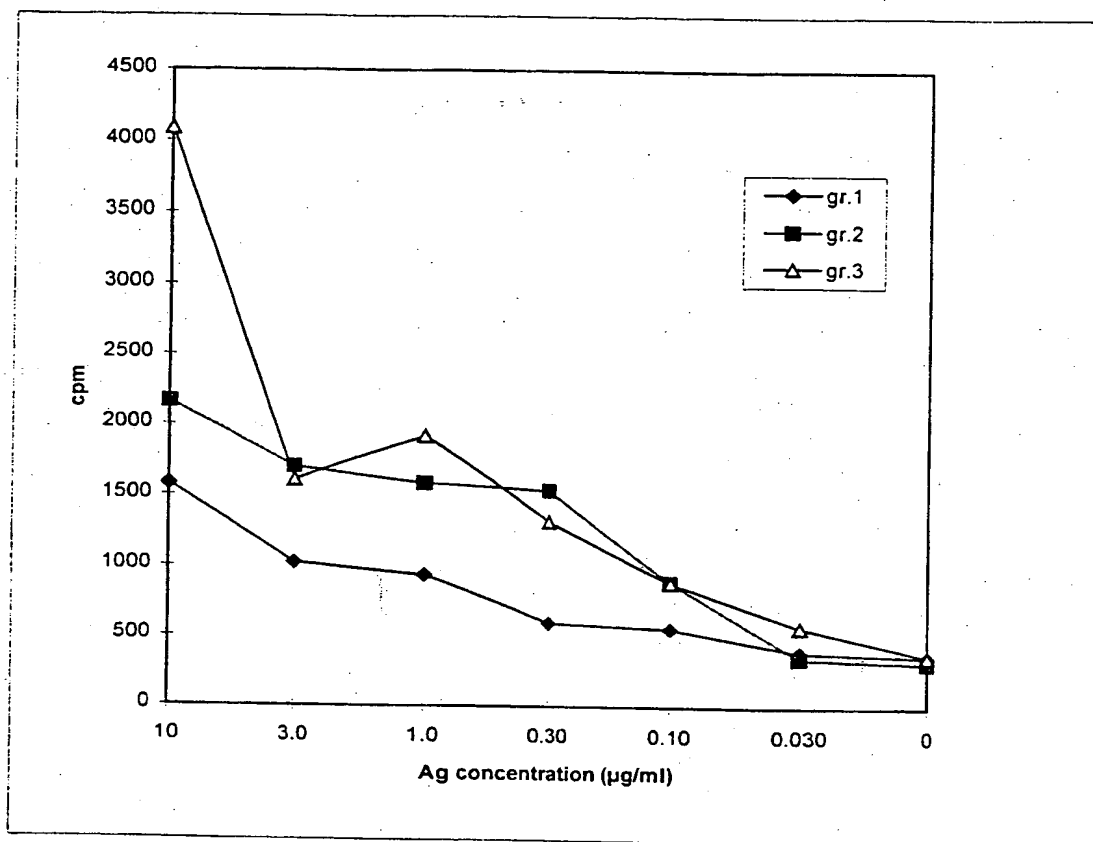
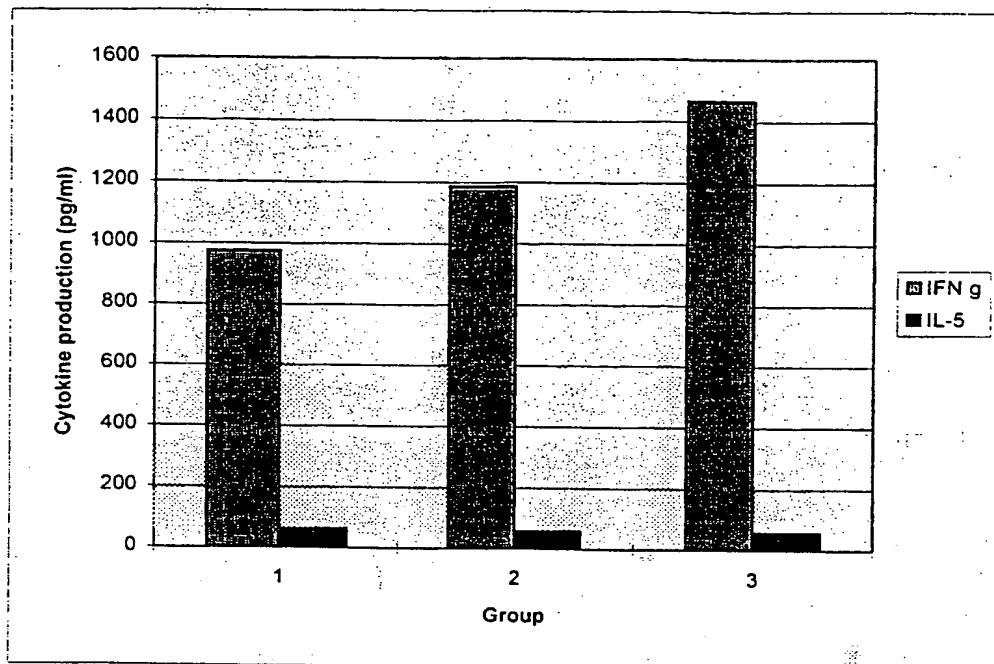


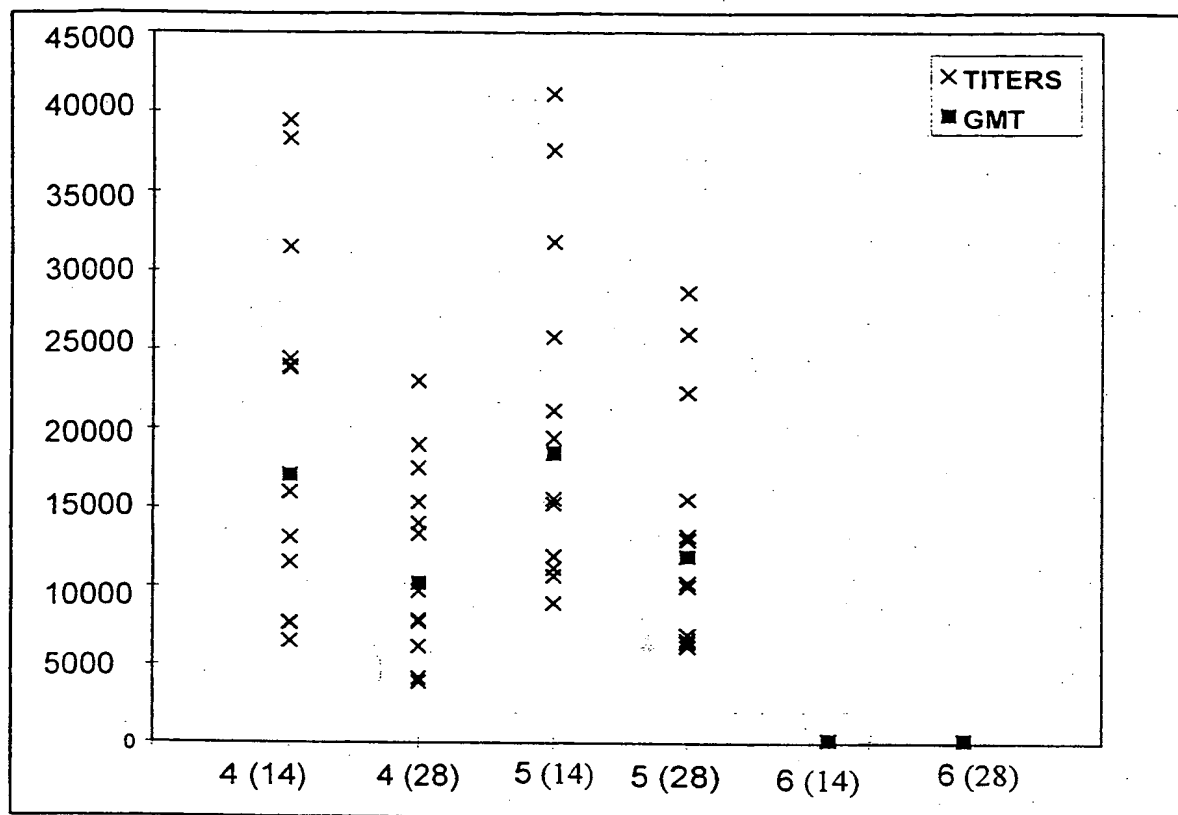
Figure 4 shows the cytokine production monitored in splenic cells restimulated with HBs.



Cytokine (pg/ml)		Gp 1	Gp 2	Gp 3
IFN g	HBsAg 10 μ g/ml	975	1187	1465
	Blank	157*	173*	173*
IL-5	HBsAg 10 μ g/ml	64*	59*	59*
	Blank	64*	59*	59*
RATIO IFNg/IL-5		15.2	20.1	24.8

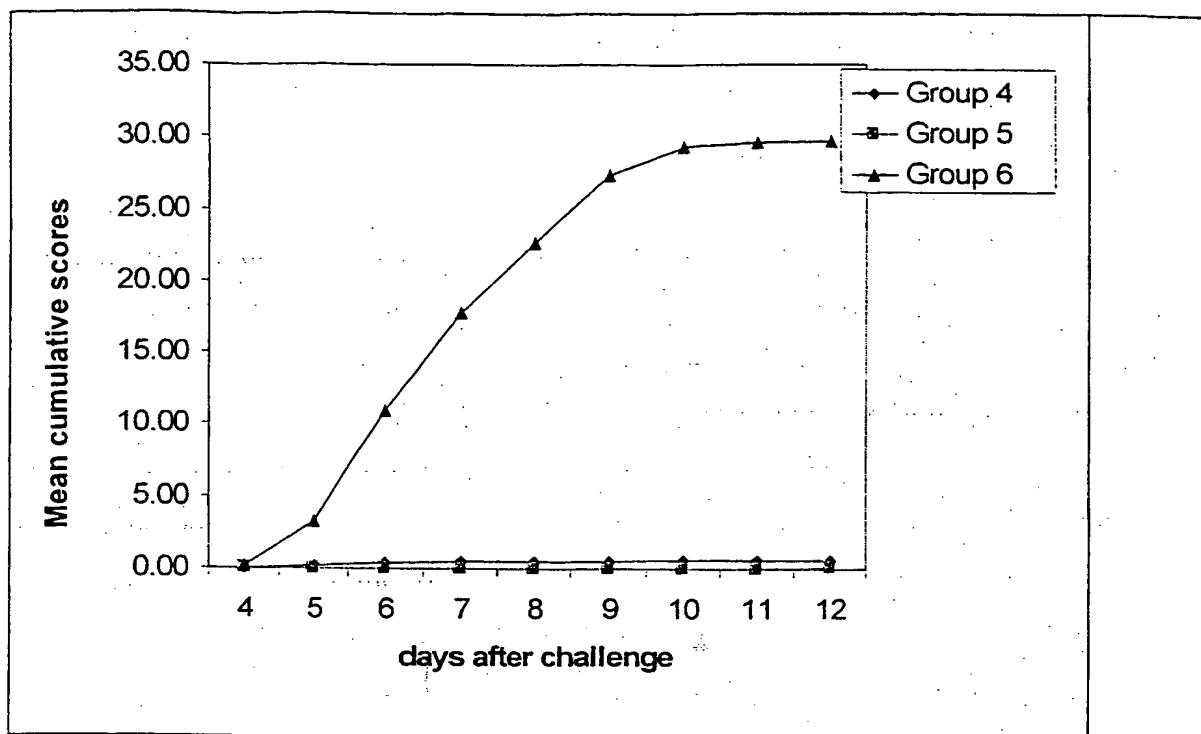
* = limit of quantification

Figure 5, Anti HSV gD titres (see example 3).



Group (Bleeding schedule)	ELISA titres	
	GMT	Average
4 (14 days post II)	17090	20336
4 (28 days post II)	10227	11825
5 (14 days post II)	18508	20903
5 (28 days post II)	11965	13796
6 (14 days post II)	200	200
6 (28 days post II)	200	200

Figure 6, Mean cumulative HSV lesion scores (see example 3)





INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : A61K 39/39, 39/00, 39/29, 39/015, A61P 31/12, 31/04, 33/06, 35/00, 37/08		A3	(11) International Publication Number: WO 00/23105
			(43) International Publication Date: 27 April 2000 (27.04.00)
(21) International Application Number: PCT/EP99/07764		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 8 October 1999 (08.10.99)			
(30) Priority Data: 9822703.6 16 October 1998 (16.10.98) GB 9822709.3 16 October 1998 (16.10.98) GB 9822712.7 16 October 1998 (16.10.98) GB			
(71) Applicant (for all designated States except US): SMITHKLINE BEECHAM BIOLOGICALS S.A. [BE/BE]; Rue de l'Institut 89, B-1330 Rixensart (BE).		Published With international search report.	
(72) Inventor; and (75) Inventor/Applicant (for US only): GARCON, Nathalie [FR/BE]; SmithKline Beecham Biologicals S.A., Rue de l'Institut 89, B-1330 Rixensart (BE).		(88) Date of publication of the international search report: 3 August 2000 (03.08.00)	
(74) Agent: DALTON, Marcus, Jonathan, William; SmithKline Beecham, Corporate Intellectual Property, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).			
(54) Title: ADJUVANT SYSTEMS AND VACCINES			
(57) Abstract <p>The present invention provides vaccine and adjuvant formulation comprising an immunostimulant and a metal salt. The immunostimulant is adsorbed onto a particle of metal salt and the resulting particle is essentially devoid of antigen.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 99/07764

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61K39/39 A61K39/00 A61K39/29 A61K39/015 A61P31/12
A61P31/04 A61P33/06 A61P35/00 A61P37/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS EPO-Internal PAJ WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 00697 A (SMITHKLINE BEECHAM BIOLOGICALS) 9 January 1997 (1997-01-09) the whole document	1-28
A	WO 96 26741 A (SMITHKLINE BEECHAM BIOLOGICALS) 6 September 1996 (1996-09-06) the whole document	1-28
A	WO 98 15287 A (SMITHKLINE BEECHAM BIOLOGICALS) 16 April 1998 (1998-04-16) the whole document	1-28
A	EP 0 812 593 A (SMITHKLINE BEECHAM BIOLOGICALS) 17 December 1997 (1997-12-17) the whole document	1-28
	-/-	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

22 May 2000

Date of mailing of the international search report

29/05/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Moreau, J

INTERNATIONAL SEARCH REPORT

Inter. Appl. No.
PCT/EP 99/07764

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, A	WO 99 48525 A (SMITHKLINE BEECHAM BIOLOGICALS) 30 September 1999 (1999-09-30) the whole document	1-28

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 99/07764

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 27 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/EP 99/07764

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9700697 A	09-01-1997	AU 695720 B	20-08-1998
		AU 6222196 A	30-12-1996
		AU 696338 B	10-09-1998
		AU 6359196 A	22-01-1997
		BG 102146 A	30-10-1998
		BR 9608612 A	04-05-1999
		BR 9609414 A	18-05-1999
		CA 2221511 A	19-12-1996
		CA 2222455 A	09-01-1997
		CN 1215337 A	28-04-1999
		CZ 9703902 A	13-05-1998
		CZ 9704189 A	17-06-1998
		WO 9640242 A	19-12-1996
		EP 0831901 A	01-04-1998
		EP 0833662 A	08-04-1998
		HU 9802199 A	28-01-1999
		HU 9900061 A	28-04-1999
		JP 11507026 T	22-06-1999
		JP 11507935 T	13-07-1999
		NO 975555 A	09-01-1998
		NO 976035 A	16-02-1998
		NZ 311000 A	29-04-1999
		NZ 312132 A	29-04-1999
		PL 323863 A	27-04-1998
		PL 324242 A	11-05-1998
		SK 176197 A	08-07-1998
WO 9626741 A	06-09-1996	AP 766 A	17-09-1999
		AU 695921 B	27-08-1998
		AU 4878096 A	18-09-1996
		BG 101851 A	30-04-1998
		BR 9607458 A	30-06-1998
		CA 2212552 A	06-09-1996
		CN 1176604 A	18-03-1998
		CZ 9702674 A	18-02-1998
		EP 0810877 A	10-12-1997
		FI 973457 A	22-08-1997
		HU 9801242 A	28-08-1998
		JP 11501018 T	26-01-1999
		NO 973873 A	22-08-1997
		NZ 302674 A	28-01-1999
		PL 321966 A	05-01-1998
		SK 115097 A	04-02-1998
		US 5972346 A	26-10-1999
		ZA 9601460 A	29-08-1996
WO 9815287 A	16-04-1998	AU 714930 B	13-01-2000
		AU 4781297 A	05-05-1998
		CN 1238696 A	15-12-1999
		CZ 9901167 A	11-08-1999
		EP 0939650 A	08-09-1999
		NO 991524 A	29-03-1999
		PL 332633 A	27-09-1999
EP 812593 A	17-12-1997	AP 515 A	09-08-1996
		AT 157882 T	15-09-1997
		AU 685443 B	22-01-1998
		AU 6426494 A	11-10-1994

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 99/07764

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 812593 A		AU 705739 B	03-06-1999
		AU 7541696 A	13-02-1997
		BR 9405957 A	12-12-1995
		CA 2157376 A	29-09-1994
		CN 1119829 A	03-04-1996
		CZ 9502467 A	13-03-1996
		DE 69405551 D	16-10-1997
		DE 69405551 T	26-03-1998
		DK 689454 T	08-12-1997
		WO 9421292 A	29-09-1994
		EP 0689454 A	03-01-1996
		ES 2109685 T	16-01-1998
		FI 954514 A	22-09-1995
		GR 3025483 T	27-02-1998
		HU 72916 A	28-06-1996
		IL 109056 A	15-06-1998
		JP 8508722 T	17-09-1996
		NO 953759 A	22-09-1995
		NZ 263538 A	28-10-1996
		PL 310598 A	27-12-1995
		SG 48309 A	17-04-1998
		SK 117395 A	06-11-1996
		US 5776468 A	07-07-1998
		ZA 9401957 A	31-01-1995
WO 9948525 A	30-09-1999	AU 3417299 A	18-10-1999

THIS PAGE BLANK (USPTO)